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A STUDY OF MICROBIAL INHIBITION IN MEAT PRODUCTS TO ACQUIRE INFORMATION FOR USE IN CONTROLLING THE GROWTH OF UNDESIRABLE MICROORGANISMS IN SUCH PRODUCTS

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S U M M A R Y

Investigations were carried out on the growth - inhibiting effect of lactobacilli, micrococci, enterococci and some other bacteria on clostridia, salmonellae, staphylococci and bacilli, as a basis for employing bacterial antagonisms to improve the quality of semi-dry sausages. Two thousand seven hundred fifty strains were isolated from cured meat and their physiological properties and antibacterial activities examined. A great percentage of the strains inhibited the growth of clostridia and enterotoxic staphylococci both in bacterial media and sausages. Some of those strains may be utilized in sausage technology. However, none of them showed an evident action on salmonellae in the environment and under conditions of sausage manufacture.

Extensive studies were carried out on methods of Salmonellae isolation from raw sausage meat and a new selective-differential medium for the isolation of bacteria causing green discoloration in sausages was devised.

I N T R O D U C T I O N

Dry or semi-dry raw sausages, depending upon their moisture content, have a relatively low pH as a major preservative factor. The taste of the average United States consumer favours relatively mild products with distinctive flavours but not to sharp /acid/, hard, or salty. A large potential, therefore, for expanded markets would appear to be in the semi-dry sausages. Unfortunately, the increased moisture

content and decreased acid and salt that would enhance the marketability of sausages also would make them more susceptible to spoilage. If spoilage types are more likely to grow, the bacteria of public health significance, such as Salmonella and some other pathogens, are likewise more likely to be found.

Findings of many authors show that raw meat that may be used for sausage manufacture may relatively frequently be contaminated with the above mentioned microorganisms. For example, salmonellae in pork were discovered in 43.7% /Felsenfeld et al., 1970/, 56% /Weissman and Carpenter, 1969/ and 80% /Shotts et al., 1961/ of samples examined. Weissman and Carpenter /1969/ found that the incidence of Salmonella in beef carcasses was 74%. Studies of some authors revealed that approximately 20 percent of the fresh pork sausage samples /Steele, 1963; Galton et al., 1954/ and 12.5% /Galton et al., 1954/ to 16.6% /Buczowski et al., 1970/ of smoked pork sausage samples were contaminated with salmonellae. Galton et al./1954/ reported Salmonella in 7.5% from national distributors and 57.5% in fresh and smoked sausage products from local processors. Goepfert and Chung /1970/ studied the behaviour of salmonellae during the manufacture and storage of Thuringer sausage. They showed that when a final pH of the product was 5.2 - 5.4, salmonellae were able to multiply in the sausage emulsion during the course of fermentation. The number of viable salmonellae in finished Thuringer sausage declined during refrigerated storage of the product. However, the rate of decline was not sufficient to

appear in meat and sausages are Cl.perfringens and staphylococci. For example, Cl.perfringens presence was showed in market meats in 60 - 70% /Hall, 1962/, in sausages 80% /Kafel, 1965/ to 100% /Mc Killop, 1959/ of samples tested. It is known that also staphylococci may frequently be found in meats. Our own investigations revealed the above bacteria in 100% of samples of cured chopped meat designed to sausage manufacture.

Some of the pathogenic and spoilage bacteria may survive or even multiply in cured meat during the production cycle of sausages. Unfortunately the application in sausage manufacture the ingredients, such as for example antibiotics that may contribute to the elimination of undesirable microflora from those products is not permitted at present.

In recent years, numerous papers have been published on interactive phenomena among microorganisms commonly present in foods. The studies presented in those papers have mainly dealt with the influence of food bacteria on growth of staphylococci /DiGiacinto and Frazier, 1966; Kao and Frazier, 1966; McCoy and Faber, 1966/ and of enterococci on certain Bacillus and Clostridium species /Kafel and Ayres, 1969/. Little information, however, is available about possible inhibitory activities of food microorganisms on salmonellae. Semi-dry raw sausages represent a special item of foods in which during the production processes numerous bacterial species always occur and in which bacterial interactions may take place. This work was undertaken with the aim of investigation of microbial inhibition in sausages, to acquire

MATERIALS AND METHODS

Elaboration of the medium for selection of antagonistic organisms

With the purpose of exclusion from further investigations the bacteria that produce hydrogen peroxide and develop green discoloration of sausages the SK medium was deviced. The formula of the medium is as follows:

Beef extract 5 g
Tryptone 10 g
Dextrose 10 g
Tween 80 0.5 ml
Sodium chloride 30 g
Potassium nitrate 3 g
Sodium nitrite 0.2 g
Monopotassium phosphate 5 g
Sodium citrate 5 g
Magnesium sulfate 0.8 g
Manganous chloride 0.14 g
Agar 15 g
Distilled water 840 ml

Dissolve ingredients, adjust pH to 6.2 and sterilize at 121°C /15 lb pressure/ for 15 min. After cooling down to 50 - 55°C add 50 ml haemolyzed blood which was formerly frozen, defrosted and warmed up to around 40°C; add also 90 ml steam sterilized /10 min./ 0.5% benzidine hydrochloride water solution. Mix and pour very thin leyers into plates. Hydrogen peroxide producing bacteria form on this medium black colonies.

Selection of antagonistic organisms

Samples of cured chopped meat assigned to sausage production were many times collected in 5 meat plants and inoculated on the SK agar. The plates were then incubated at 25°C for 72 h and colonies which by appearance resembled micrococci, enterococci, pediococci and lactobacilli were selected. A number of strains belonging to family Pseudomonadaceae were also isolated. Altogether 3,260 bacterial strains were collected.

Determination of physiological features of the strains isolated

The physiological features that may be important in sausage production were examined in all the strains selected by the following methods:

Growth capacity on common media - inoculation on nutrient agar and incubation of the plates at 25°C for 72 h.

Growth capacity under anaerobic conditions - inoculation on SK agar and incubation in anaerobic jar in nitrogen at 25°C for 72 h.

Growth in the environment containing curing salts - inoculation on SK agar and incubation at 25°C for 72 h.

Growth capacity at 8°C - inoculation on SK agar and incubation at 8°C for 10 days.

Catalase production - slide test with bacterial colonies and 10% hydrogen peroxide.

Hydrogen peroxide production - inoculation on SK agar and

the colonies.

Nitrate reduction - strains capable of growing on nutrient agar and those not growing on this medium - inoculation into nutrient broth containing 1% of KNO_3, or into APT broth with 1% KNO_3, respectively, and incubation at 25°C for 72 h; Griess test with alphanaphthylamine and sulphanilic acid.

Acid production from glucose and sacharose under aerobic and anaerobic conditions - strains capable of growing on nutrient agar and those not growing on this medium - inoculation into peptone water containing 1% glucose or sacharose, or into APT broth with these sugars, respectively, incubation at 25°C for 72 h and addition of indicator to the media.

Gelatinase production - inoculation on nutrient agar or APT agar both with the addition of 0.4% gelatine /according to Frazier/; incubation at 25°C for 72 h; test with mercuric chloride solution.

Lipolytic activities - inoculation on Staphylococcus No.110 agar containing 3% NaCl and fortified with egg yolk or on APT agar with the addition of 3% NaCl and egg yolk; incubation at 25°C for 72 h; pearly layers on the surface of colonies or precipitation zones around colonies were considered as positive results.

Coagulase production - inoculation of 8 h broth cultures into the fresh rabbit plasma; incubation of the tubes in water bath at 37°C for 3 h and then at room temperature until next day.

Testing for antibacterial action

All the collected strains were used to determine their antagonistic action toward the following test bacteria: Salmonella typhimurium, S.choleraesuis, S.dublin, Staphylococcus aureus 100 A /enterotoxic/, Clostridium perfringens A, Cl. botulinum A and B, Cl.oedematiens A, Cl.sporogenes, Cl.bifermentans, Bacillus cereus, Bac.licheniformis, Bac. subtilis and Lactobacillus viridescens. A procedure similar to that described by Jennings and Sharp /1947/ for demonstrating the antibacterial activity of staphylococci was used. A single streak of each of 2,750 collected strains was placed on the diameter of APT agar containing 0.2% glucose instead of 1.0%; and incubated at 15°C, 37°C and some strains also at 25°C for 48 h. APT agar with 1.0% glucose was also used for comparison. After growth of the primary inoculum had been assured, the plates were inoculated by streaking each of the other test organisms at right angles /i.e., across the line of growth/ to the strain examined. Then the plates were reincubated under anaerobic /clostridia/ or aerobic /other test organisms/ conditions at 37°C for 48 h. A vernier caliper was used to measure the zone of inhibition.

Testing for inhibition of spore germination

Those among the collected strains which were found to be antagonistic to the tested clostridia or bacilli were used to investigate their influence on spore germination of the above spore formers. All the spore forming bacteria, except Cl. perfringens, were grown for 24 h in cooked liver medium

was grown in Ellner's /1956/ medium. Vegetative cells were destroyed by pasteurization at 80°C for 15 min. Otherwise the streaking procedure was the same as that described for vegetative growth.

Inactivation of Cl. botulinum toxin by antagonistic bacteria.

The microorganisms which on bacterial media showed antagonistic action on Cl.botulinum were tested for their possibility of inactivation of Cl.botulinum A and E toxins. The experiments were performed on mice. Each animal of the control groups received intraperitoneally 0.2 ml of a particular dilution of the crude toxin /48 h cooked liver broth culture/ whereas the test groups of mice were injected with the same material which was formerly inoculated with an antagonistic strain and incubated at room temperature for 24 h.

Influence of catalase positive bacteria on the antagonistic activities of catalase negative organisms

One hundred of broth cultures of lactobacilli that inhibited the growth of clostridia and of some other bacteria and 100 broth cultures of enterococci showing the antagonistic activities were separately mixed together with each of 10 strains of micrococci, one strain of E.coli and with "Bactoferment" starter culture. The mixture was then inoculated

angles to the diameter, and reincubated. Zones of inhibition were measured using a vernier caliper.

Experimental sausages

Five series each of 35 semi-dry "Polish raw smoked sausages" 50 g of weight were prepared. The production formula of the sausages was as follows:

Pork - 70 kg
Pork fat 30 kg
Sodium chloride 2.80 kg
Sugar 0.20 kg
Potassium nitrate 0.10 kg
Pepper 0.15 kg
Marjoram 0.05 kg
Garlic 0.05 kg

The sausage mass was stuffed in natural casings with a diameter of around 30 mm. Afterwards, the product was kept at 6°C and relative humidity 85 - 90%. Then it was smoked at 20°C for 24 h and made partly dried at 12°C for 3 days. After stuffing in casings 2 ml of the mixture of bacterial suspension were injected into the center of each sausage. The suspensions were made by rinsing off the bacterial colonies from agar cultures with physiological saline solution and adjusting the density of the suspensions to a required concentration.

By this means, into every sausage around 5,000 000 cells per 1 g of an antagonistic bacterium and around 10,000 per g of a test microorganism were introduced. Three strains of

acidophilus and 3 other lactobacilli, all chosen from among the bacteria that on bacterial media showed relatively highest antagonistic activities, were applied. The test bacteria used /salmonellae, clostridia, staphylococci/ were the same as in the testing for antibacterial action in artificial media. Control groups /the same amount as the experimental groups/ of the sausages were inoculated with the test bacteria only.

Bacteriological examination of the sausages was carried out one day after completion of the sausage production. Each entire sausage was homogenized, dilutions made and quantitative examination by plate count method performed using Baird-Parker agar /1963/ and nutrient agar.

The 3-tube Most Probable Number /MPN/ procedure was employed for enumeration of salmonellae and Clostridia. The media used here were tetrathionate bile brilliant green broth - brilliant green agar, and cooked liver broth - lactose egg yolk agar, respectively.

Average numbers of bacteria from 5 series were calculated.

R E S U L T S

S e l e c t i o n o f a n t a g o n i s t i c b a c t e r i a

Altogether 9,260 bacterial strains were isolated from raw cured meat. Two thousand seven hundred fifty of them were, according to their physiological features, classified

they did not produce proteolytic or lipolytic enzymes, were coagulase negative, did not produce at all or produced only a small amount of hydrogen peroxide and possessed some other positive features. Those strains represented the following groups of bacteria: Micrococcus - 1,780, Lactobacillus - 320, Pediococcus - 45, Enterococcus - 160, Pseudomonas - 100, strongly unclassified bacteria 345 strains.

Testing for antibacterial action

The number of strains inhibiting the growth of the bacteria tested is shown in Table 1.

Table 1

Number of strains examined	Number of strains inhibiting the growth of the bacteria tested in bacterial rash			
	Salmonellae	Clostridia	Bacilli	Staphylococci
Micrococci 1,780	18	-	7	2
Lactobacilli 320	11	264	16	298
Pediococci 45	-	4	-	-
Enterococci 160	-	160	13	-
Pseudomonads 100	-	-	4	-
Unclassified 345	1	93	42	70
Total 2,750	29	521	126	370

In connection with the figures presented in Table 1 it should be pointed out that: the inhibitory action of micrococci on salmonellae was very weak and those bacteria lost their antagonistic properties after several months from the time of their isolation; the zones of inhibition of salmonellae

L.acidophylus which inhibited the growth of salmonellae within the range of 15 mm; however, all the lactobacilli influenced the growth of salmonellae only in AFT agar with 1.0% glucose but not with 0.1% or 0.2% of this sugar; the only antagonistic strain from the unclassified group which exerted the antibacterial action on salmonellae showed this property in all the media used in the experiments; this strain could rather not be used in sausage manufacture because in experiments carried out on chickens it caused intestine and joint troubles; great majority of lactobacilli considered in the investigations were evidently antagonistic for enterotoxic staphylococci and clostridia; when the antagonism of some strains collected against bacilli took place it was evidently less expressed in comparison with the inhibition of clostridia.

Inhibition of spore germination

All the strains examined which antagonistically influenced the growth of vegetative cells of clostridia or bacilli, inhibited their spore germination in the same ranges. The zones of inhibition were, however, 1 – 2 mm wider.

Investigations on the possibility of inactivation of C.botulinum toxin by the antagonistic bacteria

In the investigations 10 such strains each of lactobacilli

No significant inactivation of Cl.botulinum A and B toxin was discovered.

Antagonistic activities of mixed catalase positive and catalase negative cultures

It was found that lactobacilli and enterococci in mixtures with catalase positive organisms such as micrococci, "Bactoferment" starter culture, or B.coli, inhibited the growth of Bac.subtilis and Bac.licheniformis. However, they did not show any action on clostridia and staphylococci whose growth was inhibited by individual cultures of those antagonistic bacteria.

Bacterial antagonism in experimental sausages

The results are presented in Table 2.

Table 2

organisms reduced into sausages	Antagonistic bacteria						
	Lactobacilli				Enterococci		
	L.acid.	L 1	L 2	L 3	E 1	E 2	E 3
	Number of test organisms in 1 g of experimental and control sausages						
typhimurium	303 210	243 376	297 318	117 257	190 185	190 178	387 420
choleraesuis	79 108	105 176	120 88	75 120	81 212	95 65	160 135
dublin	93 110	64 160	66 76	123 115	134 95	83 122	105 105
Ph. aureus	15,800 16,150	250 7,880	135 5,330	875 10,670	7,800 8,810	13,200 10,170	9,630 12,360
perfringens	0 6	0 4	0 5	0 11	0 0	0 11	0 9

Upper figures= experimental sausages with the antagonistic and the test bacteria

lower placed figures = control sausages with the test bacteria only

D I S C U S S I O N

Numerous investigations have been devoted to the problem of antagonism among various bacteria and the research was also dedicated to the utilization of this phenomenon in food manufacture. However, little is known about the controlled inhibition or the elimination of disease producing bacteria, such for example as salmonellae, from their food products throughout the application of antagonistic bacteria. It is obvious that such antagonistic organisms should be able to grow under conditions existing during the manufacture of the product taken into consideration. They should also possess possibly greatest number of physiological features which will contribute to the development of desirable flavour and aroma of the product.

Raw semi-dry sausages represent such an item of foods in which disease producing bacteria, for example salmonellae, Cl.perfringens and Staph.aureus frequently occur and in which bacterial interaction could be utilized.

Among 2,750 strains examined only 25 of them inhibited the growth of salmonellae in bacterial media. The inhibitory zones, except 2 strains, were rather insignificant and some strains lost their activities after several month laboratory storage. Unfortunately, one of those two strains /L.acidophilus/ very poorly grew at low temperatures and the other one /E.coli/ appeared to be harmful to the experimental animals. Therefore, their usefulness in practice seems to be questionable. Bacilli considered in the present work were also rather inconsiderably influenced by the antagonistic

was evidently inhibited by a high proportion of the strains examined. No correlation was found between the inhibition of Cl.botulinum growth and the toxin inactivation by antagonistic bacteria.

Antagonistic lactobacilli and enterococci inhibiting the growth of clostridia or staphylococci did not act on these bacteria when growing in a mixture with catalase positive organisms. Therefore it is supposed that hydrogen peroxide may play a role in the mechanism of the antagonism.

In the experiments carried out on sausages the antagonistic bacteria did not show any significant action on the growth or survival of salmonellae and the results obtained are in general consistent with those in bacterial media. On the basis of all findings of the present work it seems that bacteriological strains antagonistic for salmonellae and simultaneously capable of growing in the environment of raw sausages unlikely occur in cured meats. Goepfert and Chung /1970/ also stated that salmonellae can survive the fermentation process in sausages and that the sliced vacuum-packed product remained salmonella-positive throughout the 42 day holding period at various temperatures.

Antagonistic action of three strains of the lactobacilli used in our experiments contributed evidently to the decrease of number of enterotoxic staphylococci in sausages but enterococci did not show any noticable influence on these microorganisms. It may be observed, however, that enterococci, similarly as lactobacilli, did not favour the survival of Cl.perfringens in the product.

may be noticed between the interactive phenomena among the microorganisms in sausages and in bacterial media whose [illegible] composition and conditions of their incubation [illegible] in some degree similar. All the antagonistic bacteria isolated in the investigations on sausages, according to the bacteriological examination, did not decrease the organoleptical value of the products, nor did they show any disease symptoms in animals. It may be supposed that although these bacteria did not result in the elimination of salmonellae, their [illegible] activities against clostridia or staphylococci may be utilized in the manufacture of sausages.

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Investigations on Various Enrichment Methods and Incubation Conditions for Isolation of Salmonellae from Sausage Meat

It is difficult to isolate salmonellae from a material containing a small number of these bacteria and contaminated heavily with many other microorganisms. This was confirmed by many authors who showed that in some cases isolation of salmonellae from sausage meat or other materials was unsuccessful although they contained these microorganisms /W.H.O. Bull., 1968/. A suitable selection of cultural media is very important for Salmonella isolation. It is generally known that on cultural examination of meat samples slightly contaminated with salmonellae selective enrichment media are of great importance and among them commonly used are tetrathionate and selenite broths.

Taking into consideration that there are now some 1300 Salmonella serotypes it may be supposed that the authors of the media have never tested their value in relation to all the serotypes. In the available literature there are reports indicating that some cultural media deviced for Salmonella isolation do not secure the detection of some Salmonella serotypes in samples tested. So, Hobbs /1963/ found that on testing egg powder containing a small number of other microorganisms it was more easily to isolate salmonellae in a nutrient broth than in selective media. Thompson /1955/ tesitng feaces samples obtained better

results using a nutrient broth than a selenite broth. On cultural examination of egg powder Galton et al. /1961/ isolated more often salmonellae using a nutrient broth in comparison with tetrathionate broth.

Selective enrichment media used for Salmonella isolation may exert a harmful effect on some Salmonella serotypes. Smith /1952/ found that the selenite and tetrathionate broth were toxic for S.choleraesuis and S.abortus ovis. Leifson /1936/ demonstrated toxic properties of the selenite broth on S.choleraesuis and Danwart and Ayres /1953/ made a similar observation in respect to the influence of the tetrathionate broth on S.paratyphi A. A toxic effect of the selenite broth on S.typhimurium, S.abortusovis, S.typhisuis, S.newport, S.rostock, S.enteritidis and S.montevideo was shown by Kafel /1964/.

Besides a chemical composition of the above media, a great influence on the results of examination may have also temperature and time of their incubation. In the majority of laboratories a temperature of 37°C is commonly used.

However, Harvey and Thompson /1953/ and Georgala and Boothroyd /1965/ obtained better results applying 43°C. They found that this temperature inhibited to some extent the growth of E.coli but it did not affect harmfully that of salmonellae. Spino /1966/ isolated regularly salmonellae from surface waters in the tetrathionate and selenite broths incubated at 41.5°C but no growth was obtained at 37°C. However, McCoy /1962/ found that the tetrathionate broth

salmonellae. In different laboratories, cultural media are incubated for 12 to 96 h. However, the results of investigations carried out by some authors have shown that a twofold subculturing from a selective enrichment medium incubated for different time gave more positive results than a single subculturing. In such investigations subcultures were made usually after 24 and 48 h or 24 and 72 h.

On cultural examination for salmonellae of a material heavily contaminated with other microorganisms Jameson /1961, 1962/, McCoy /1962/, Galton et al. /1961/ used the reinoculation method. In this method samples tested were inoculated into an enrichment selective medium and incubated, next reinoculation was made into the same medium and after incubation subculture was made on a differential selective agar. The authors obtained better results by this method than using the same media without reinoculation.

Results of examination of a material containing a small number of salmonellae depend on many other factors such as a relation between quantity of the material and medium, chemical composition of samples, their pH, fat content, Salmonella serotype, kind and number of other microorganisms present in the sample, and so on.

As it was mentioned above, the methods used up to now for the detection of salmonellae in food products do not guarantee always positive results although these bacteria are present in the samples tested. Although in the world literature there are many reports on this problem no

results is yet available. Experimental research works concerned mainly single or only a few Salmonella serotypes and only some methodical variants were simultaneously used. In the last few years, the incidence of salmonellae in animals and breeding centres is increasing throughout the world, these bacteria are isolated more often from food products, especially meat, and the number of food infections caused by these bacteria in humans increases constantly. These difficulties and the insufficience of the present methods for Salmonella isolation encouraged us to undertake these investigations which aimed to work out or to select the best methods for isolation of salmonellae from cured chopped sausage meat.

MATERIALS AND METHODS

Preparation of meat samples.

The material examined consisted of chopped sausage meat samples. They were taken randomly under normal production conditions. Each 100 g sample of the meat was mixed carefully with 300 ml of a saline solution and the suspension obtained was filtered through a filter paper. The filtrate was checked for the presence of salmonellae and inoculated with Salmonella serotype examined /10 cells per one g of the filtrate/. Samples contaminated naturally with salmonellae were not taken into consideration in evaluating the results. Before inoculation the filtrate was checked quantitatively for the presence of other microorganisms

was always greater then 10.000.000 bacterial cells per 1 ml.

P r e p a r a t i o n o f S a l m o n e l l a
s t r a i n s.

Unpublished Salmonella strains were obtained from the Strain Collection, Department of Microbiology, Veterinary Institute, Puławy, Poland. They were inoculated into nutrient broth, incubated at 37°C for 18 h and 10 - fold serial dilutions in Ringer's solution were prepared. The number of salmonellae in each dilution was determined on nutrient agar by Koch's plate method. Until further use the bacterial dilutions were stored at 8°C. Basing on the number of colonies grown on agar plates, sausage meat filtrates were inoculated with appropriate bacterial dilution so that 1 g of the filtrate contained 10 Salmonella cells. The following Salmonella serotypes were used: S.typhimurium, S.abortusovis, S.brandenburg, S.choleraesuis, S.newport, S.enteritidis, S.reading, S.pullorum, S.anatum, S.london, and S.senftenberg. Tests with all the serotypes were repeated twelve times, except those with S.anatum which were made 4 times.

Alltogether 124 experiments were carried out and 48 methodical variants in every experiment were applied.

I n o c u l a t i o n o f c u l t u r e m e d i a
w i t h m a t e r i a l e x a m i n e d a n d
i n c u b a t i o n c o n d i t i o n s.

Ten ml of each enrichment selective medium was inoculated

Table 1

Culture media and methods of their incubation

Variant number	Culture medium	Incubation method
1	SB	37°/24 h
2	"	37°/48 h
3	"	37°/24 h, R-SB-37°/24 h
4	"	37°/24 h, R-TB-37°/24 h
5	"	37°An/24 h
6	"	37°An/48 h
7	"	37°An/24 h, R-SB-37°An/24 h
8	"	37°An/24 h, R-TB-37°An/24 h
9	"	43°/24 h
10	"	43°/48 h
11	"	43°/24 h, R-SB-43°/24 h
12	"	43°/24 h, R-TB-43°/24 h
13	"	43°An/24 h
14	"	43°An/48 h
15	"	43°An/24 h, R-SB-43°An/24 h
16	"	43°An/24 h, R-TB-43°An/24 h
17	TB	37°/24 h
18	"	37°/48 h
19	"	37°/24 h, R-SB-37°/24 h
20	"	37°/24 h, R-TB-37°/24 h
21	"	37°An/24 h
22	"	37°An/48 h
23	"	37°An/24 h, R-SB-37°An/24 h
24	"	37°An/24 h, R-TB-37°An/24 h

Variant number	Culture medium	Incubation method
26	TB	43°/48 h
27	"	43°/24 h, R-SB-43°/24 h
28	"	43°/24 h, R-TB-43°/24 h
29	"	43°An/24 h
30	"	43°An/48 h
31	"	43°An/24 h, R-SB-43°An/24 h
32	"	43°An/24 h, R-TB-43°An/24 h
33	BB	37°/24 h
34	"	37°/48 h
35	"	37°/24 h, R-SB-37°/24 h
36	"	37°/24 h, R-TB-37°/24 h
37	"	37°An/24 h
38	"	37°An/48 h
39	"	37°An/24 h, R-SB-37°An/24 h
40	"	37°An/24 h, R-TB-37°An/24 h
1	"	43°/24 h
2	"	43°/48 h
3	"	43°/24 h, R-SB-43°/24 h
4	"	43°/24 h, R-TB-43°/24 h
5	"	43°An/24 h
6	"	43°An/48 h
7	"	43°An/24 h, R-SB-43°An/24 h
8	"	43°An/24 h, R-TB-43°An/24 h

Explanation:

SB - selenite F broth /Leifson, 1936/:

BB - bile broth;

37°C/24 h - incubation at 37°C for 24 h;

An - incubation under anaerobic conditions in a mixture of butane and propane;

R - reinoculation of 0.15 ml of one medium into another.

After incubation subcultures were made on brillant green agar plates /BG/, which were then incubated at 37°C for 24 - 48 h. From each plate 5 colonies of appearance typical for Salmonella were transferred on triple sugar iron agar /TSI/. With the material grown on the above medium the slide agglutination test was performed using polyvalent H serum, group sera for somatic antigens and sera for individual flagellar antigens.

R E S U L T S

The results are presented in Fig. 1 - 3.

D I S C U S S I O N

E f f e c t i v e n e s s o f e n r i c h m e n t s e l e c t i v e m e d i a.

As may be seen from Fig. 1 the greatest number of positive results was obtained successively using TB, then SB and finally BB media. In addition to the kind of enrichment selective media used, a great effect on the results of examinations had also incubation conditions discussed below.

effective than for 24 h although salmonellae were found more often after 24 h incubation at 43°C under aerobic conditions. With TB medium more positive results were obtained after 24 h than after 48 h at 37°C but the number of isolates at 43°C was reverse. In BB medium the greatest number of isolates was obtained always after 48 h. These data show that incubation time of SB and TB media may effect differently the results of Salmonella isolation depending on the presence or lack of the atmospheric oxygen.

I n c u b a t i o n t e m p e r a t u r e.

Salmonellae were isolated more often almost in all the media incubated at 43°C. The only exception was SB medium incubated for 48 h in which less positive results were obtained at 43°C than 37°C.

E f f e c t o f a n a e r o b i c i n c u b a t i o n.

In SB medium salmonellae were isolated evidently more frequently under anaerobic than aerobic conditions. Similar results were obtained in BB medium, except 48 h incubation at 43°C. However, in TB medium the greatest number of isolations was obtained always under aerobic conditions.

R e i n o c u l a t i o n s i n t o s e l e c t i v e m e d i a.

The results obtained by different combinations of reinoculation and those obtained with single inoculations of media incubated for 24 h are compared in Fig. 2. These

the number of Salmonella isolations in comparison with single inoculation of the media, but in other variants the results obtained by the reinoculation method were worse. The best combination of liquid media for the reinoculation technique proved to be SB medium with reinoculation into TB medium. However, none of the reinoculation variants used equalled in results to the method of inoculation of TB medium incubated at 43°C for 48 h under aerobic conditions. The inoculation of BB medium and subsequent reinoculation into SB medium gave even distinctly worse results than inoculation of any single medium. In addition to media combination, a significant effect on the results of reinoculation had also incubation conditions.

Relationship between the number of positive results and Salmonella serotype.

The material examined was inoculated always with the same number of salmonellae to obtain 10 bacterial cells in 1 ml of the sausage meat filtrate. However, divergent results shown in Fig. 3 were obtained with different Salmonella serotypes. For instance, S.london was isolated 317 times and S.abortusovis only 6 times per 576 possible positive results. Some Salmonella serotypes were isolated more easily from a given selective enrichment medium than from other media. S.abortusovis was isolated only from TB broth, similarly as S.pullorum, but S.newport was discovered

Fig. 1.

Influence of Enrichment Media and Conditions of their Incubation on Isolation Frequency of Salmonellae

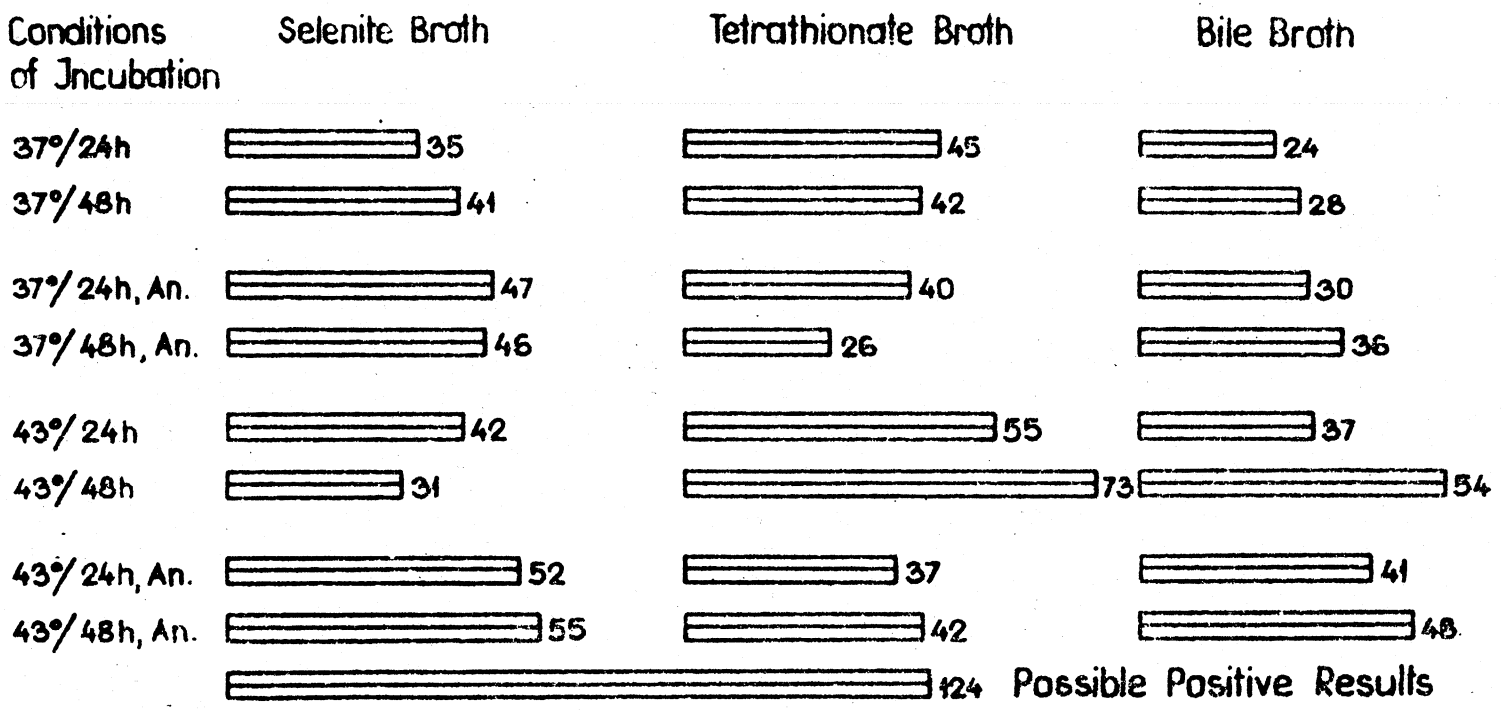
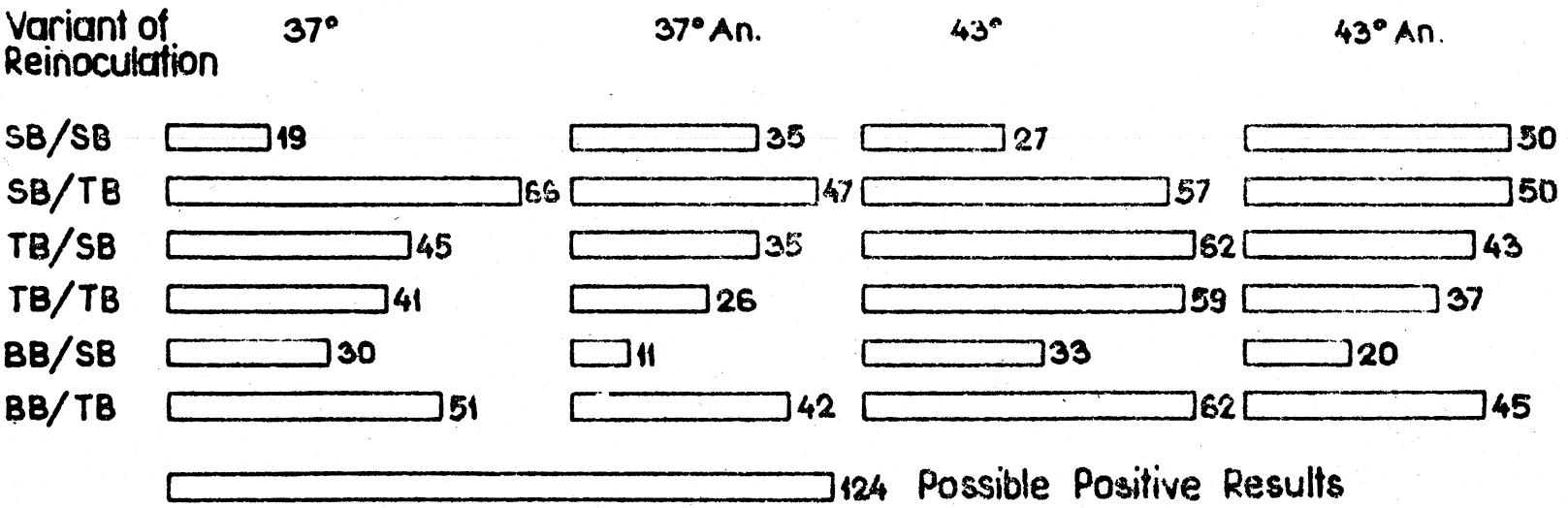


Fig. 2.

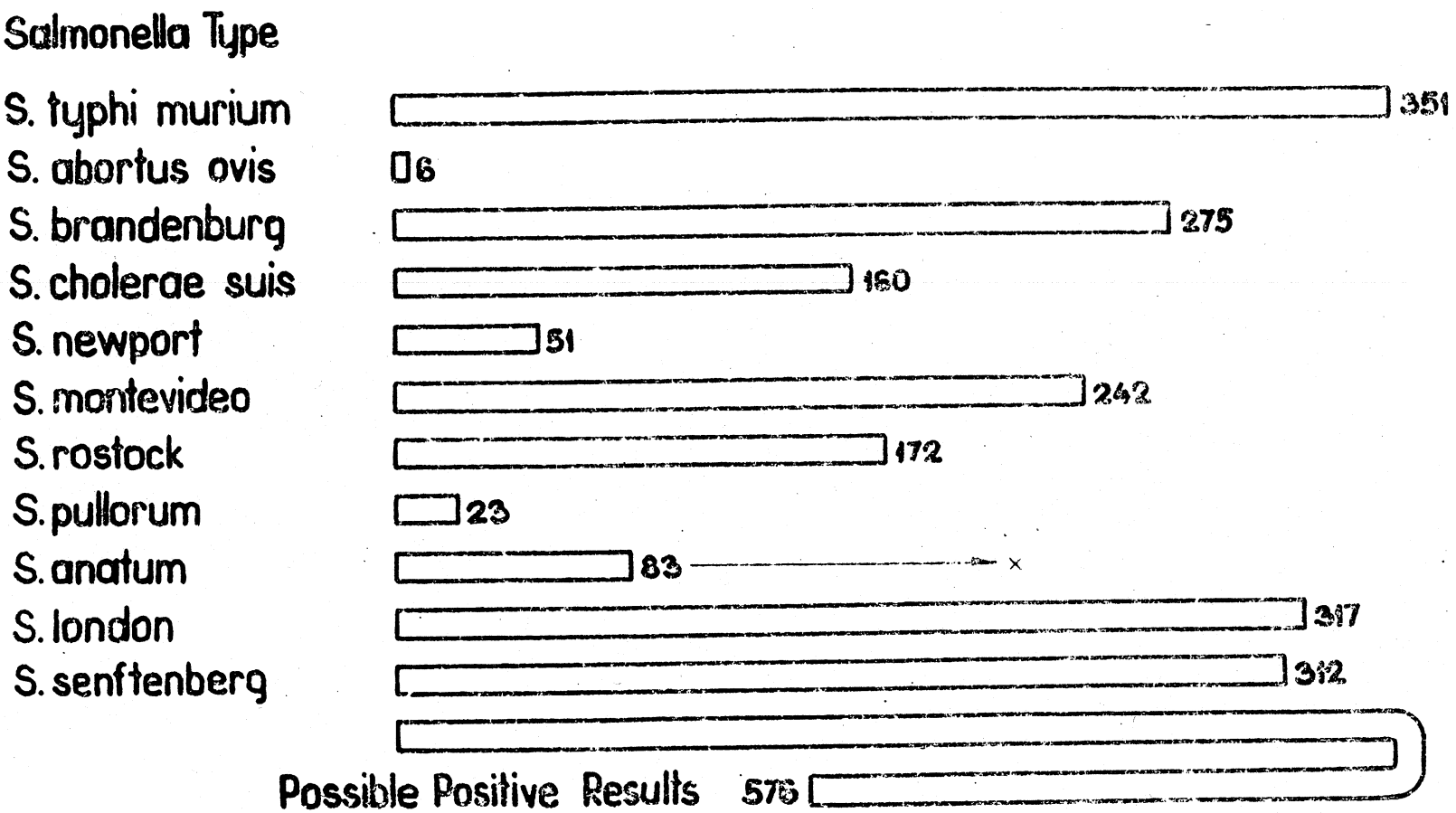
Number of Positive Results Obtained from Various Reinoculations of Enrichment Media Incubated under Different Conditions



SB = Selenite Broth; TB = Tetrathionate Broth; BB = Bile Broth

Fig. 3.

Frequency of Positive Results at the Examination of the Same Number of Samples Containing 10/g Salmonellae and 10 milion/g Other Bacteria



A g e n e r a l a n a l y s i s o f t h e r e s u l t s o b t a i n e d.

Summarizing all the results obtained, it may be stated that the greatest possibilities of Salmonella isolation from the sausage meat haevily contaminated with other bacteria gave successively the following methodical variants:

1. Variant 26 - TB medium incubated at 43°C for 48 h /73 positive results/.

2. Variant 4 - SB medium incubated at 37°C for 24 h, with subsequent reinoculation into TB broth incubated at 37°C for 24 h /65 positive results/.

3. Variant 27 - TB broth incubated at 43°C for 24 h with subsequent reinoculation into SB broth incubated under the same conditions /62 positive results/.

4. Variant 44 - BB medium incubated at 43°C for 24 h with subsequent reinoculation into TB broth incubated under the same conditions /62 positive results/.

The remaining combinations of media and incubation conditions gave usually less objective results. Salmonellae were isolated the most rarely using BB medium incubated at 37°C for 24 h under anaerobic conditions with subsequent reinoculation into SB medium incubated identically as BB medium. Using this variant, a twice smaller number of positive results was found than that obtained by inoculation of BB medium without subsequent reinoculation.

A detailed analysis of all the results show that different combination of selective enrichment media with incubation

results. Therefore, fundamental principles that the best results can be obtained in each case using a given medium, incubation time, temperature, anaerobic conditions or reinoculation may not be generalized. This depends also on many other factors as Salmonella serotype, kind of contaminating microflora, chemical composition of a tested sample, its weight and so on.

The results of this section of our Project may be summarized as follows: among 3 enrichment selective media tested, the greatest possibility of Salmonella isolation from meat haevily contaminated with other microorganisms showed tetrathionate bile brilliant green broth incubated at 43°C for 48 h. Selenite F broth and bile broth gave less objective results; the prolonged incubation time up to 48 h and increased temperature up to 43°C may exert favourable or unfavourable effect on the number of positive results dependably on the remaining methodical parameters; anaerobic incubation of selective enrichment media had a favourable effect on the number of Salmonella isolates in selenite F broth and bile broth and an unfavourable effect in tetrathionate bile brilliant green broth; reinoculation method may increase or decrease the number of positive results dependably on other methodical parameters. However, in no case this method was better than the use of single tetrathionate bile brilliant green broth incubated at 43°C for 48 h.

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